MULTI-FACTORIAL TREATMENT PARADIGM DETECTION COULD BE THE ANSWER TO COMPLEX DISEASES: A CASE STUDY OF ALS

A Thesis
Presented to
The Academic Faculty

by

Tyler Kittel

In Partial Fulfillment
of the Requirements for the Degree
BS in Biomedical Engineering with the Research Option in the
School of Biomedical Engineering

Georgia Institute of Technology
December of 2017

MULTI-FACTORIAL TREATMENT PARADIGM DETECTION COULD BE THE ANSWER TO COMPLEX DISEASES: A CASE STUDY OF ALS

Approved by:

Dr. Cassie Mitchell, Advisor
Department of Biomedical Engineering
Georgia Institute of Technology

Dr. Robert Butera
School of Biomedical Engineering
Georgia Institute of Technology

Date Approved: December 14, 2017

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

AUTHOR CONTRIBUTIONS

Tyler Kittel: data aggregation, quality control, statistical analysis, data visualization, results interpretation, and drafting of manuscript.

Renaid Kim: data aggregation, ontology scheme development; assistance with data protocol.

Albert Lee: assistance with data aggregation, quality control, and statistical analysis.

Tian Zhang: data aggregation, data protocol assistance, computer programming.

Thao Bach: assistance with data visualization.

Mitchell lab biocurators: over 300 students have participated in data curation for the SOD1-G93A database.

Dr. Cassie Mitchell: project development, project oversight, project mentoring, analytical protocol development, assistance in results interpretation, assistance in manuscript writing.

SUMMARY

Amyotrophic Lateral Sclerosis (ALS) is a debilitating neurodegenerative disease with no known cause or cure. Through a combination of an open market-space, scientific curiosity, and the humanitarian motivation to advance medicine, every treatment option imaginable has been attempted for this condition in the hopes of finding a cure. This analysis incorporates 5026 paired treatment-to-control data points of the G93A SOD1 mouse model, the most commonly tested ALS model, in an effort to organize the vast amount of published data in the field and evaluate which approaches are most promising for further experimentation. An ANOVA analysis was completed comparing nine different pathophysiological treatment approaches to ALS as a function of seven stages of disease progression throughout the entire lifespan of the mice. Treatment efficacy was evaluated based on how well the treatment improved three disease metrics compared to their own experimental control. Patterns emerged for overall disease benefit, as well as for the three modality assessments including onset delay, survival prolongment, and general health scores. Combination treatments that fit into more than one category also performed better than individual therapies later in life. Interestingly, most treatments were administered before disease onset, yet benefit was almost solely found post-onset. These results suggest that patients may benefit from a targeted pro-active combinatorial treatment approach to combat the multiple failed regulations and general homeostatic instability characteristic of ALS. Such a conclusion correlates with the trend towards complex personal medicine treatment plans in other multifactorial diseases.

INTRODUCTION

Amyotrophic Lateral Sclerosis was unfamiliar to the general public until the ALS Association's Ice Bucket Challenge in 2014 brought attention to this debilitating and invariably fatal disease. While the social media phenomenon raised money and awareness, ALS is still as deadly and far from a cure as ever: only one FDA approved drug manages to extend patient survival by a matter of months, but the negative side-effects make its benefits controversial (*1*). The problem lies in the inherent pathophysiological complexity of this neurodegenerative condition. Familiarly occurring in only 10% of cases, no clear etiology has been identified, but rather a combination of proposed factors could explain how ALS sporadically befalls 90% of patients (*2*).

While all ALS mouse models experience the same multifactorial issues, which are also shared across many other motor-degenerative diseases, the G93A SOD1 (G93A) familial model is most commonly tested. Treatments spanning everything from melatonin (*3*) to bee venom (*4*) have been evaluated. This vast range of approaches has previously been organized into 9 defined categories based on which pathophysiological function ontology the treatment intends to improve (*5*). A prior examination of these categories has demonstrated oscillating relationships between the processes, suggesting that the diseased system experiences a homeostatic instability when trying to overcome one problem, but overcompensating and aggravating a different one (*6*). Bearing this dynamic and ever-changing condition in mind, all of these previous treatments may have failed due to only managing one ontological instability when multiple factors need to be addressed simultaneously.

Despite extensive effort to find the exact cause and treatment for ALS as well as other complex diseases, they remain a mystery to the field. One potential explanation for this elusion might be that many researchers only consider one aspect of the disease, and therefore only attempt to treat a fraction of the problem. Our study compares the treatment effect of all 9 ALS ontological treatment approaches over 7 time spans throughout the G93A mice lifespan. By including all of the major instabilities in the entire disease progression with such a large dataset, this analysis has a high chance of catching any relations that exist. Following an all-encompassing analysis, the benefit of all 9 approaches will be determined specifically for their effectiveness in delaying onset, improving health status, and prolonging mice survival time.

Correlating the multifactorial nature of complex diseases is a multiple treatment approach. The idea of targeted polytherapy is nothing new; personalized combination treatments have demonstrated clinical success in cancer patients (*7*). Recent clinical studies have shown the potential of adapting this approach into other diseases, including ALS (*8, 9*). However, the question becomes how to find the best combinations when there is already a struggle to find one single beneficial treatment?

In an effort to answer this question, evaluating and comparing the treatment efficacies of previous studies could narrow down the options. Our assessment of the most promising ALS treatments as a function of time could contribute to the creation of a more concrete therapeutic strategy for disease management: the most promising results can be combined for future preclinical experiments and the successful ones eventually adapted to clinical trials.

LITERATURE REVIEW

Due to its inherent complexity, no cure has yet been found for amyotrophic lateral sclerosis (ALS). Only 5-10% of patients have an affirmative family history for ALS, thus the remaining 90-95% ALS patients are sporadic cases with unknown etiology (*10*). Multiple studies have proposed additional factors potentially associated with ALS besides genetics, including environmental factors and pre-existing disease or conditions (*11*). In recent years, more researchers have acknowledged that ALS is, in fact, a multi-factorial pathology, involving the interplay of several pathogenic pathways that propagate the spread of motoneuron death (*12*). The principal experimental model utilized to study ALS pathophysiology, the SOD1 G93A transgenic ALS mouse model, has a precisely known mutation and yet, the exact initiating disease mechanisms remain unclear. However, thousands of experiments have revealed a multitude of factors that are contributing to disease progression. These complex interactions in the multi-factorial ALS pathophysiology have been simulated in temporal, multi-scalar computational models, which discovered the presence of mathematically unstable dynamics, or system instabilities. Despite innate compensatory regulation, the system instability and insufficient regulation prevents the re-establishment of homeostasis post-ALS onset, an observation dubbed as "homeostatic instability" (*13*). In short, ALS is not a one-factor disease but rather, a system-level instability. Future treatment strategies must embrace a holistic approach that extends beyond the search for an autonomous single root cause responsible for all cases of ALS but instead, focuses on the re-establishment of system homeostasis-- irrespective of the initiating cause.

1.1 Modality assessment can distort apparent treatment efficacy

Hundreds of journal articles have published results assessing potential therapeutic strategies in the SOD1 G93A mouse model, but unfortunately, there is no universal standard to assess level of benefit. The quantifiable data may evaluate anything from onset tremors to grip strength to survival. Even an outcome seemingly as simple as "survival" may have more than 15 experimental definitions. Some preclinical treatment approaches are cited to be particularly effective in delaying ALS onset, others in slightly prolonging survival, and still others in improving standard post-onset health status. Infrequently does a study examine all such outcomes, and if it does, even more rarely is the benefit conferred across all outcome assessment modalities. For example, treatment targeting inflammation is generally cited as delaying onset, but appears to be less effective if treatment is initiated in later disease stages (*14*). Another example is apoptosis, where a study showed that blocking apoptosis towards end-stage conferred no prolonged survival benefit, but it did improve the quality of life scores of the mice (*15*). Conversely, another study showed that treatments targeting axonal transport are only effective in prolonging survival (*16*). Interestingly, axonal transport deficiency occurs very early in the disease progression (*17*), with the anterograde mitochondrial transport most affected by the SOD1 G93A mutation.

Thus, it is evident that treatments may be more beneficial in improving only survival, only time of onset, only quality of life scores (e.g. rotarod performance, grip strength, body weight, etc.), combinations thereof, or none of the above (*18*). Further research into which preclinical assessments are most reliable for ultimately predicting clinical relevant efficacy will lead to universal standards for experimental assessment modalities.

However, in the meantime, the specific outcome metric utilized as an experimental assessment modality must be considered when comparing treatment efficacy among studies.

1.2 Combination therapy is the future of ALS treatment

Combination therapy treatments are those categorized as targeting more than one pathophysiological treatment category. Within the literature, dual-treatment usage such as rasagiline and riluzole (*19*), minocycline and creatine (*20*), lithium and Neu2000 (*21*), and Minocycline + riluzole + nimodipine (*22*), have either extended survival, delayed onset, or accomplished both more effectively than either of the individual treatments dispensed alone. A polytherapy approach to such a complex disease could potentially help with treating the multiple factors involved in ALS instability; it is unlikely that one drug can tackle all necessary pathways.

A personalized combination therapy approach was first introduced in cancer patients where it has repeatedly demonstrated clinical success in the oncological field. Randomized, placebo-controlled clinical trials for ALS patients using combination therapy, such as minocycline-creatine and celoxicob-creatine, have largely outperformed individual therapies in slowing disease progression in patients (*23*). The latter two treatment concepts were adapted from the results of a murine model experiment, demonstrating the potential value and applicability of preclinical models.

While a combination treatment approach could provide more benefit to the patient, it will take time to determine which combinations are both safe and effective. The SOD1 G93A mouse model is a good preclinical starting point, as the results from this model are directly applicable to familial SOD1 G93A ALS cases; although ALS

corresponding to the SOD1 G93A mutation does not precisely align with sporadic ALS, they do share many of the same pathophysiological deficiencies (*24*). Using Riluzole as the first part of a combination may help expedite a successful clinical combination therapy, considering it already has FDA approval, owing to the fact its efficacy and safety profile is well established (*25*).

1.3 Project Scope

The long-term goal of the present study is to jump-start the process of selecting the most viable potential drug combinations by imploring a large sample size, determining the impact of temporal dynamics/disease stage on treatment efficacy, and considering the assessment modality utilized to determine efficacy. This study utilizes multi-treatment meta-analysis to assess individual treatment effectiveness in improving disease parameters throughout the various stages of disease progression of SOD1 G93A transgenic mice. A large-scale aggregated analysis can ameliorate discrepancies in the literature and provide justification for logistical planning of future studies. Finally, an assessment of the most promising ALS treatments as a function of time and assessment modality will provide a foundation for the eventual creation of concrete and personalized combination therapeutic strategy for disease management--one that compensates for the dominant instability occurring at each diseases stage and anticipates the next one as the dynamics of the pathophysiology evolve.

MATERIALS AND METHODS

1.4 Study Design

In an effort to evaluate and compare the treatment efficacies of previous studies, this analysis of the most promising ALS treatments as a function of time and assessment modality could contribute to the creation of a more concrete therapeutic strategy for disease management.

1.5 Inclusion/Exclusion Criteria

3412 publications were found from a PubMed search in July 2016 with "Amyotrophic Lateral Sclerosis" or "ALS" and "mouse" or "G93A" in the title or abstract (*5, 6*). These articles were downloaded using e-journal subscriptions available at the libraries of Georgia Institute of Technology and Emory University. Treatment information and results were systematically entered into a relational database using FileMaker Pro 14 Advanced and underwent rigorous quality control procedures (*7*).

Of these downloaded articles, 1232 had recorded quantifiable measures of mouse health and were identified for inclusion. Of this subgroup, 324 tested SOD1 G93A ALS mice, and 291 of those were treated with the intention of ameliorating disease conditions. So that treatments from each individual paper could be compared based on performance relative to control, consistency between mice age and disease stages was also an inclusion criterion. Only articles with high copies of SOD1 G93A-B6SJL-Tg ALS mice, SOD1 G93A-B6Cg-Tg ALS mice, mice with unstated genetic background, or B6SJL mice backcrossed with other genetic backgrounds with disease progression timelines consistent

with the high copy B6SJL mice and genotype-matched controls were included. The final constraint for inclusion required that articles must have data spanning at least two disease stages, so that the effect of treatments over the duration of disease remained the focus instead of the effectiveness at alleviating symptoms at a certain point. 199 published articles met all criteria and were included in the analysis.

1.6 Treatment Category Definitions

All treatments were organized into one of the nine pathophysiological categories based on their target approach to helping the mice (Table 1). Systemic, which is the largest and most varied category was further divided into subcategories for analysis.

Table 1 – Treatment categories, definitions, and examples used in this analysis

Category	Definition	Examples
Apoptosis	Repair aberrant apoptotic pathways or inhibit pro-apoptotic pathways	zVAD-fmk, AEOL 10150, p75 knockout
Axonal Transport	Repair deficit in both anterograde and retrograde axonal transport	noscapine or anti-NRP1
Chemistry	Repair mishandling of metal or an imbalance in vital nutrients	DP-109, DP-460, Iron chelator VK-28, magnesium pidolate
Energetics	Primarily target mitochondrial dysfunction and use of bioenergy (ATP, glucose)	uridine, metformin, methimazole
Excitability	Repair or prevent excitotoxic damage through various mechanisms	riluzole, caffeine, progesterone, cannabinoid
Inflammation	Modulate the inflammation process	inhibition of transglutaminase 2, rofecoxib
Oxidative Stress	Lower the free radicals and oxidative stress levels	riboflavin and pramipexole
Proteomics	Repair or prevent protein aggregation.	guanabenz and resveratrol
Systemic	Considered to have an effect at a systemic level, rather than cellular or subcellular level. Subdivided into: cell therapy, diet, exercise, growth factor, gene therapy, transplant, and unconventional	Stem cell therapy, light therapy, fibroblast growth factors

Polytherapy treatments were defined as treatments that belong to two or more of the nine general categories. Most of these were combinatorial treatment regimens that were applied simultaneously (creatine and rofecoxib or celecoxib (*45*)), but if a single treatment was considered to have effects on multiple categories (e.g. bee venom on apoptosis and inflammation (*4*)), then it was also classified as polytherapy. On the other hand, if two treatments of the same category were applied (e.g. PRE-084 and resveratrol in proteomics (*59*)), then it was classified as monotherapy.

1.7 Disease Metrics

Quantifiable data points were categorized into 3 groups of assessment modalities to investigate the effect on certain outcomes compared to others. Onset indicators included measures such as probability of onset, paralysis onset, onset age, etc. Survival signs included survival rate, age, cumulative survival, etc. Health status included the rest, comprising of a variety of metrics ranging from body weight to rotarod performance to grooming frequency.

1.8 Normalization

Each treatment data point was paired with a time-matched control from within the same article to create paired data points. Treatment effects were assessed by dividing the quantified outcome of a disease metric from the treated group by that of the control group to determine the fold of change in the mice condition caused by the treatment within the pair. Treatment effects resulting in values less than 1, those that performed worse than the control group, were excluded from the main analysis and only included in the harmful treatment sub-analysis.

1.9 Time Bin Selection

The binning process provides a detailed assessment of different treatment categories' effectiveness for each stage throughout the entire lifespan of the mice. Thus, the number of bins was selected as enough to capture significant trends while still retaining long term features of the dataset, such as the slight drops in average treatment effectiveness at pre-onset and mortal stages of disease. Each bin was required to hold approximately the same number of total data points to maintain comparability.

All treatment-control paired data points were divided into 7 bins according to the mice age in days (Table 2). These bins correspond to and are grouped according to the disease stages of the mice model at that time: 3 before onset, onset, and 3 after onset, given that the average onset time for the untreated G93A SOD1 transgenic mouse model is 99.27±1.79 days for mixed sex high copies B6SJL groups (*6*).

1.10 Statistical Analysis

Statistical significance of treatment ontologies was assessed with an ANOVA with post-hoc Tukey test for each time bin; a p-value of 0.05 considered the threshold for significance. To mitigate the bias from any one paper, each included study was weighted equally regardless of individual sample sizes of mice studied. All statistical tests were conducted in MATLAB R2014a software. Figures were generated in IGOR Pro.

1.11 Results Layout

In order to demonstrate trends found for specific temporal, ontological, and assessment modality relationships, the main ANOVA results will be presented in three

different ways: first by disease stage, second by ontology, and third by disease metric. References will be made in one section to the others to reiterate the data is the same no matter the organization strategy.

RESULTS

1.12 Summary of Data Sources Used

After filtering the database for established inclusion & exclusion criteria, 199 papers were included in this meta-analysis, for a total of 5026 normalized data points (Table 2). These data points were further broken down into one or more of nine treatment categories, and into one of seven age groups correlating to the disease progression within the mice model chosen. The distribution of data per ontology found for this analysis corresponds with previous systematic review findings (*5*): systemic and inflammation papers are most common, while axonal transport is the least published in the field.

Table 2 – Sources Overview: 5026 individual points were included in this analysis.

	Apoptosis	Axonal Transport	Chemistry	Energetics	Excitability	Inflammation	Oxidative Stress	Proteomics	Systemic	All
Number of articles	19	3	15	40	27	31	32	17	62	199
Total number of data points	434	39	188	1183	682	713	791	321	1120	5026
0-70	77	4	42	218	115	77	182	31	185	867
71-85	39	7	26	161	108	85	101	29	83	418
86-100	79	7	40	228	163	130	121	66	204	963
101-110	60	1	23	129	87	114	78	45	165	640
111-120	81	11	281	202	120	124	113	65	187	853
121-130	48	2	13	113	39	108	111	43	146	563
131+	50	7	14	129	50	75	89	42	150	552
Onset	37	0	37	73	24	40	83	17	59	356
Health Status	253	19	99	831	543	446	443	133	627	3024
Survival	144	21	68	279	174	227	265	171	434	1645

When comparing the time of onset and death between treated and control mice in the 9 ontologies, excitability treatments were most effective at delaying onset, around

9.09 days. While oxidative stress was mediocre at best for onset, this category was the most effective at prolonging average survival at 14.21 days. Chemistry treatments indicated the least survival improvement in comparison with others (Figure 1).

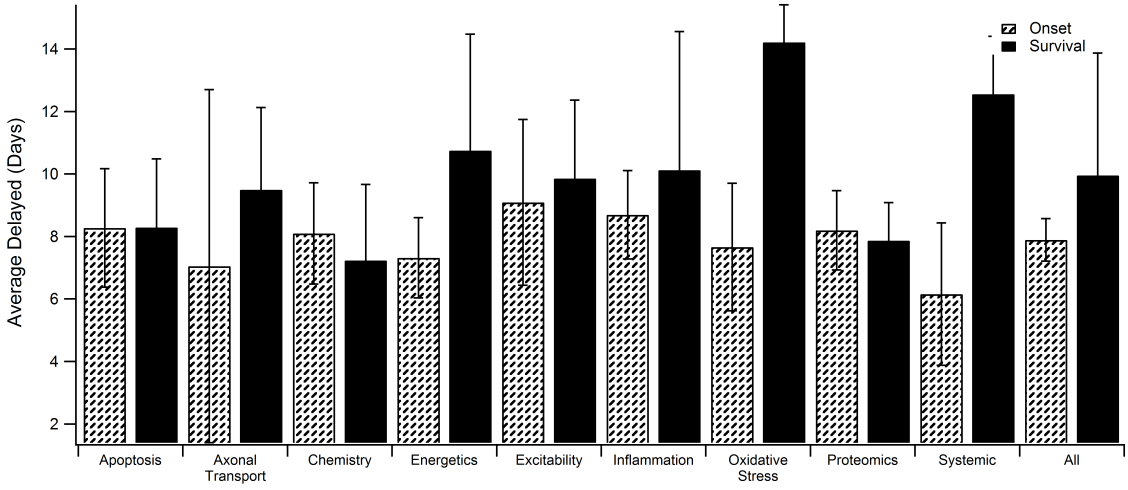


Figure 1 – Average delayed onset and survival data for each ontology. ANOVA analysis with post-hoc Tukey test determined no significant differences between ontologies for either onset or survival delay.

1.13 Most treatments were administered pre-onset

Across all ontologies, reported treatment start dates were collected and analyzed (Table 3). While the largest normalized treatment effects occurred post-onset, most treatments were administered in early life (Figure 2). The mean start dates were between 41.72 and 54.22 days of age, which is well before the 100-day onset-mark for this mouse model. A Kruskal-Wallis and multiple comparison test yielded no significant differences between the treatment start dates of any of the ontologies. Considering that these treatments began pre-onset, and most of the effects happened post-onset, there is potentially a delayed effect taking place.

	Apoptosis	Axonal Transport	Chemistry	Energetics	Excitability	Inflammation	Oxidative Stress	Proteomics	Systemic	All
Control Onset Average	101.4	101.3	99.1	110.7	99.7	102.9	103.4	96.5	102.1	101.3
Treated Onset Average	105.4	108.4	106.1	118.1	107.2	111.8	105.9	104.7	104.8	108.7
Difference Average	8.3±1.9	7.1±5.7	8.1±1.6	7.3±1.3	9.1±2.7	8.7±1.4	7.7±2.0	8.2±1.3	6.2±2.3	7.9±0.7
Control TOD Average	128.9	127	127.5	131.5	131.8	130.4	132.7	131.5	135.5	131.3
Treated TOD Average	137.6	135.2	128.7	138.4	144	139.8	135.7	141.3	143.6	138.9
Difference Average	8.3±2.4	9.5±3.7	7.2±2.5	10.7±4.4	9.9±1.2	10.1±1.2	14.2±1.9	7.9±3.9	12.6±3.4	10.0±1.0
Mean Treatment Start Date	44.1±8.8	52.2±11.5	50.9±7.5	45.0±6.5	53.24.5	54.2±3.8	48.4±4.7	41.7±5.1	48.6±4.1	49.5±1.8

Table 3 – Mean ontology start dates. Onset, survival and start date of treatment data summary table for each ontology. The mean start dates of all ontologies were around 49.5 days of age, while the standard errors were around 1.818 (n = 6-122).

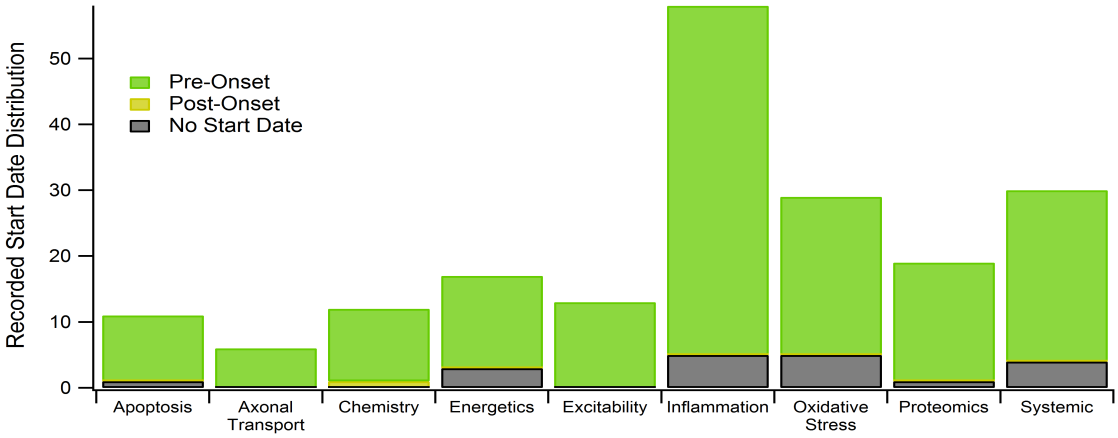


Figure 2 – Paper reported start dates demonstrate that very few (0-5.7%) treatments were administered post-onset in any treatment ontology.

1.14 Treatment Category Efficacy Changes Throughout Disease Progression

All 5026 data points were included in an ANOVA analysis with a Post-Hoc Tukey test evaluating every category's normalized treatment efficacy in every age group throughout disease progression in all assessment modalities (Figure 3).

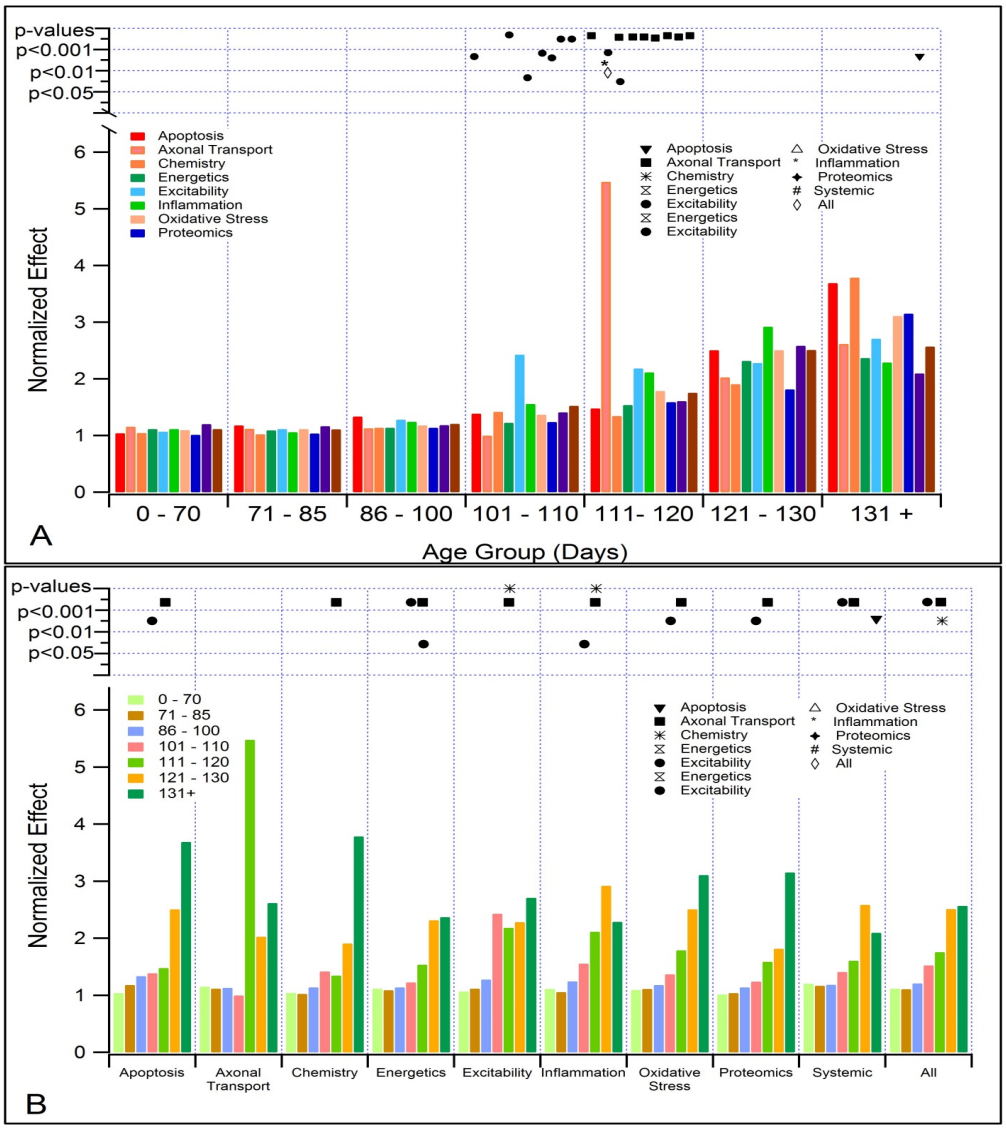


Figure 3 – Treatment category efficacy changes throughout disease progression. Organized by A) disease progression and B) individual treatment category. 0-100 days: No one treatment performs significantly better prior to onset. 101-110 days: excitability treatments have the best effect around disease onset. 111-120 days: axonal

transport performs the best immediately following onset. Chemistry is significantly worse than a few other categories. 121-130 days: no trends emerge during the later disease stage. 131+ days: Apoptotic treatments are significantly more effective than systemic at end-stage. $p < 0.05$.

1.14.1 1-70 days

When taking into account all disease measures, no treatments were notably more effective during the youngest age range (Figure 3). Within the specific assessment modalities, no significant relationship occurred for improving survival (Figure 6) or health status (Figure 5) metrics; the only significant relationships were apoptosis outperforming a few other treatments in delaying onset symptoms (Figure 4).

1.14.2 71-85 days

While the trend of no significant benefit for all measures (Figure 3) and health status (Figure 5) continues, there are some benefits for the other two assessment modalities. Apoptosis is almost twice as effective as any other treatment in delaying onset (Figure 4) and systemic displays significant benefit to the mice in prolonging survival characteristics (Figure 6).

1.14.3 86-100 days

While apoptosis had the most benefit for all measures (Figure 3) and health status (Figure 5), no category of treatments performed significantly better than another in the stage immediately preceding onset for all disease measures (Figure 3) as well as any metric sub-analysis (Figure 4-6).

1.14.4 101-110 days

Analyzing all disease modalities, excitability treatments demonstrated a noticeably greater effect than all others (Figure 3); this was mostly due to its effects on the health status modality at this stage (Figure 5). There was an observed benefit from this treatment for onset delay, but inflammation was slightly better in this stage (Figure 4). Systemic continued the trend of being slightly better than others in survival, but overall all treatments have relatively the same benefit to the mice (Figure 6).

1.14.5 111-120 days

Axonal transport treatments were exceedingly more effective than every other category on all modalities combined (Figure 3). This occurred solely from this category's ability to extend the survival of the mice significantly more than any other (Figure 6). Chemistry was especially ineffective in this stage (Figure 3).

1.14.6 121-130 days

There was no observable trend in any of the nine categories. However, most treatment categories showed at least twice the benefit in this stage compared to control data (Figure 3).

1.14.7 131 days and after

Chemistry, which had been consistently one of the least beneficial, had the highest effect at the end. Interestingly, this category's efficacy during this stage came solely from prolonging survival (Figure 6), as it was the worst at improving health status measures

(Figure 5). Apoptosis was closely behind on all measures (Figure 3), and much more effective at health status (Figure 5).

1.15 Treatment Category Efficacy Varies by Ontology

Overall, the apoptosis treatment category efficacy steadily increases in normalized treatment effect to control as the disease progresses; culminating in being one of the best categories at end-stage (Figure 3) due to its benefit for the health status and survival modalities at that time. This ontology also demonstrated a significantly higher benefit than all other treatments for delaying onset (Figure 4).

While axonal transport had the least amount of data and was subsequently excluded from some assessment modality analyses, this category displayed the highest treatment effect immediately post-onset of any other category at any other age group due to its profound benefit in improving survival signs (Figure 6).

Chemistry was close behind apoptosis for a steadily increasing treatment effect as the disease progressed (Figure 3); this category's most notable benefit was extending end-stage survival better than all other treatments (Figure 6).

Energetics peaked in treatment effect during later disease stages (Figure 3), but was only once the best treatment for any assessment modality at any time, during a post-onset stage in survival (Figure 6).

Excitability performed slightly above average during the early stages, but was significantly better than all others during the typical time of disease onset (Figure 3). This benefit is almost solely from the comparative improvement for health status disease metrics

(Figure 5).

Inflammation gave an average benefit to the mice during early-life, but had one of the worst overall effects at end-stage. This treatment did have the highest effect of all ontologies in delaying onset at the 101-110 and the 111-120 days groups (Figure 4), but was not effective at prolonging survival (Figure 6).

Oxidative stress usually fell in the middle of the categories and was never found to perform the best of any time or modality beyond the onset modality at the 121-130 days range.

Proteomics consistently ranked in the bottom of the categories for all measures, until its high effect at end-stage (Figure 3). Within the onset sub-analysis, it had almost the same effect as inflammation at the 101-110 days range, but showed a sharp decline in the next bin and then did not have enough data beyond the 111-120 days group to assess its effect on onset.

Systemic treatments were slightly more effective than other treatments during early-life and, though not usually the best, consistently performed as one of the best categories in each modality throughout disease progression.

1.16 Treatment Category Efficacy Varies by Disease Metric

1.16.1 Onset

When analyzing the normalized treatment effect of each category, a sub-analysis of just the measurements relating to onset provide more specific insight into how each

treatment is affecting the mice (Figure 4). Apoptosis had the most beneficial effect in early life, despite not indicating any significance in the main analysis (Figure 3). This treatment continues to stand out in the next stage of disease progression, and is found to have almost twice the benefit at the 71-85 age group than any other treatment. Its effect diminished just prior to onset: inflammation, excitability, and proteomics were the top three categories, but there were no significant differences in the 86-100 age range. Inflammation performed the best in the 101-110 days age group for delaying onset, and retained this status in the 111-120 days group; proteomics was a close second in the former and apoptosis in the latter. While neither was significant, oxidative stress performed the best right before end-stage, and systemic at the end-stage 131+ days age group. Axonal transport did not have enough data to be included in this assessment modality, and several categories were excluded due to lack of data in the post-onset stages.

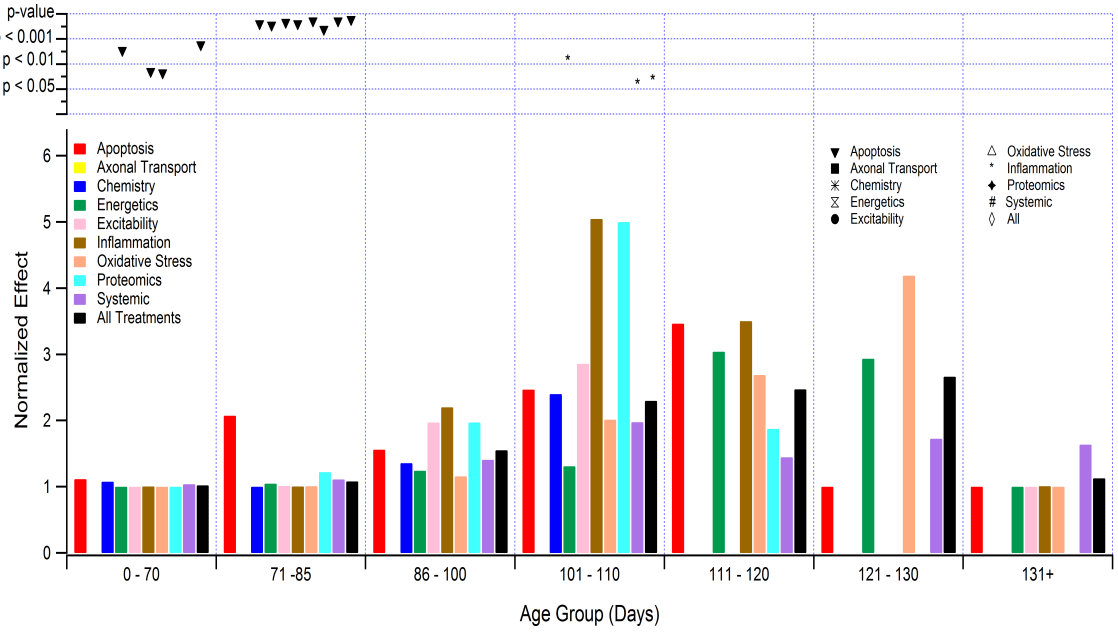


Figure 4 – Analysis of onset assessment measures by category. Most notably, apoptosis is significantly more effective than all other categories at delaying onset symptoms during early life. $p < 0.05$.

1.16.2 Health Status

All measurements not related to onset or survival were placed in the health status assessment modality and analyzed separately. While apoptosis consistently showed a high treatment effect during every pre-onset stage, correlating with the onset modality results, no significant trends emerged until the high benefit of excitability at the 101-110 day stage. This remarkable effect is very different to the treatment's performance in the onset and survival analysis. While excitability's benefit remained high until end-stage, inflammation was best at 121-130 days, and then axonal transport and apoptosis at the 131+ day stage. Every category contained enough data to be analyzed at each stage (Figure 5).

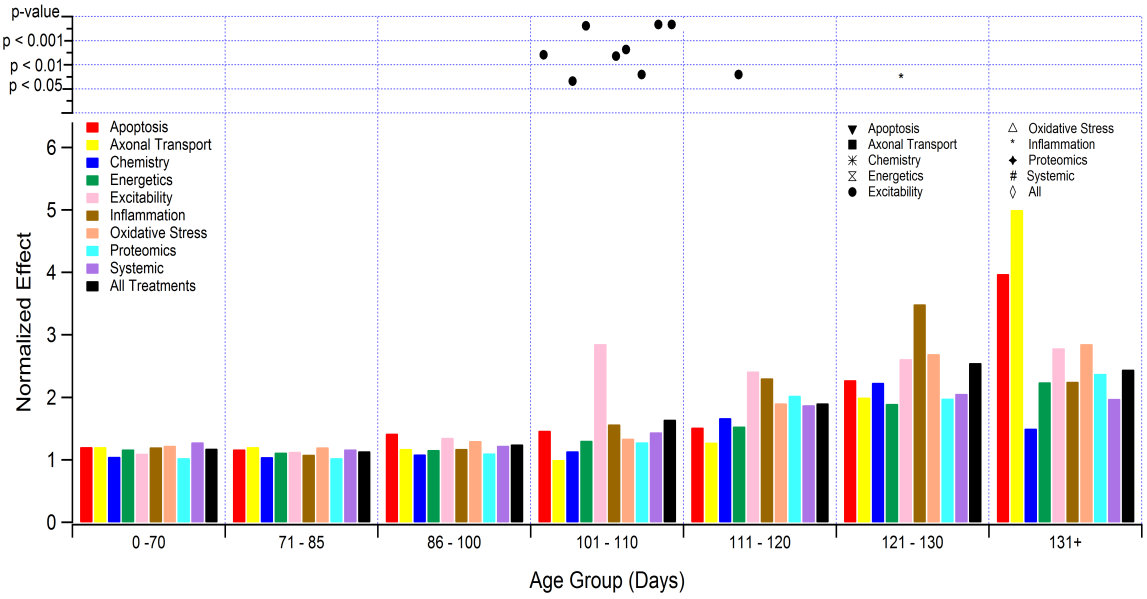
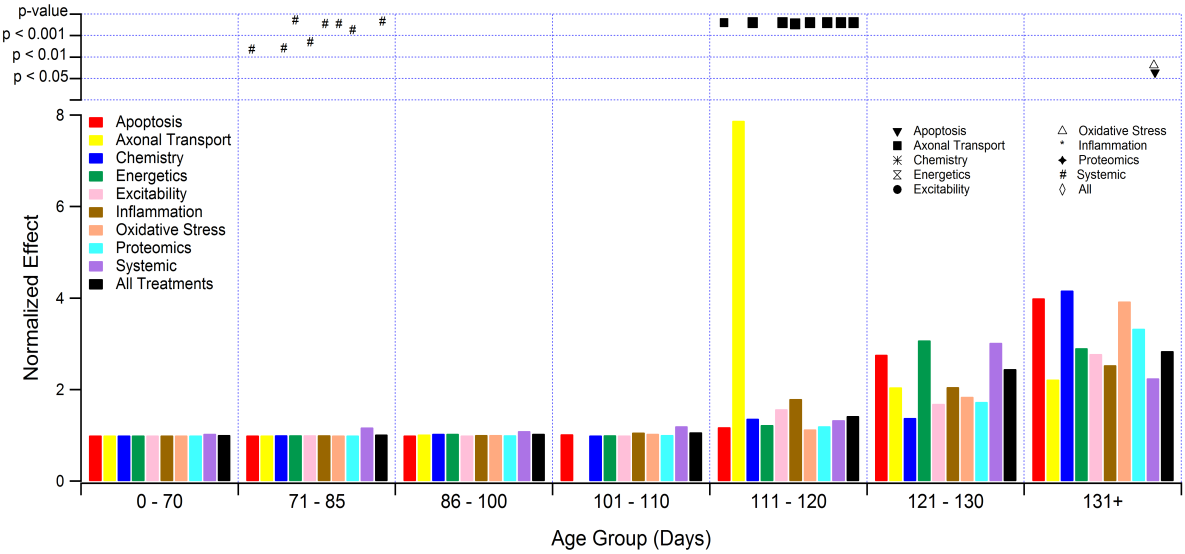


Figure 5 – Analysis of only health status measures by category. Most notably, excitability was most beneficial at disease onset, while axonal transport and apoptosis were best at end-stage. $p < 0.05$.

1.16.3 Survival

Those measurements pertaining to the survival assessment showed no correlation to the onset or health status modalities. The treatment effects of all groups were similar from

early-stage until post-onset; systemic always performed the best in these age groups, and even demonstrated a significantly better effect than all others in the 71-85 days age group.



Axonal transport had a treatment effect of almost 8, the highest of any in the entire analysis, at the 111-120 days age group. In the later disease stages, no significant trends emerged beyond systemic being the worst at prolonging survival, but the effects of each treatment were much higher than at early life (Figure 6).

Figure 6 – Analysis of survival assessment measures by category. While all were relatively ineffective in early stages, systemic was consistently the best. Post-onset, axonal transport had a substantially larger effect on mice aged 111-120 days. $p < 0.05$.

1.17 Systemic treatment subcategories also demonstrate trends throughout disease progression

The systemic treatment category warranted a sub-analysis, not just because it was the group that contained the largest number of data points, but also due to this ontology encompassing a wider range of treatment modalities and containing treatments that are most studied to date (Figure 7).

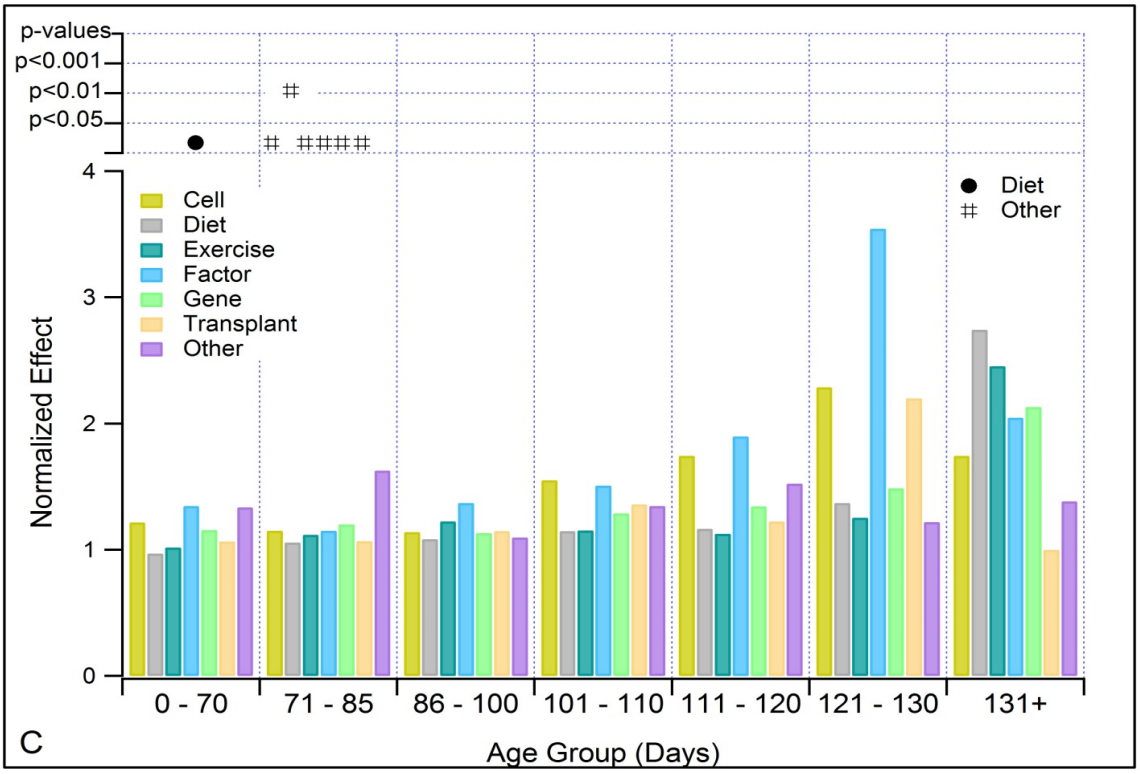


Figure 7 – Systemic treatment subcategories have widely varied treatment effects at each disease stage. Most notably, growth factors consistently performed better than other subcategory at early life and again at onset until just before end-stage. The unconventional category was significantly better in early stages of life. $p < 0.05$.

1.17.1 1-70 days

Growth factors had the highest treatment effect of all systemic subcategories, performing significantly better than diet. The others category was a close second.

1.17.2 71-85 days

Treatment effects of most categories were comparable to those of the previous age group, yet growth factors' effect fell to the average. The others subcategory indicated significantly better effects than the rest.

1.17.3 86-100 days

All treatment effects remained relatively low, but growth factors became the most effective category again.

1.17.4 101-110 days

Growth factors' benefit to the mouse remained the same, while all other categories performed better. The cell therapy category had the highest treatment effect within this age group.

1.17.5 111-120 days

Growth factors once again yielded the highest treatment effect, with cell therapy also showing relatively high benefit to the mice compared to control.

1.17.6 121-130 days

Growth factors reached its highest treatment effect in this age group. Exercise and unconventional were the worst at this stage.

1.17.7 131 days and after

While not demonstrating any statistically significant relationships, the diet subcategory had the highest treatment effect at end stage after being consistently towards the bottom before this. Exercise, another subcategory that had not previously performed well, was almost as beneficial to the mice.

1.18 Some treatments could potentially harm mice

Disease measures with a normalized value of <1 were excluded from the main

analysis of treatment effectiveness and were compared separately. A total of 1350 negative treatment points were detected (as opposed to 5026 positive included in the main analysis). A few significant relationships were found, most notably between proteomics and several other treatments in the 86-100 days age groups; the only other relationships found were between systemic and a few other treatments at 111-120 days. Most of these harmful treatment values were isolated instances of one data point where control outperformed treatment, but the treatment effect would rise to >1 at the next measured time point and again be classified as beneficial. However, 48 out of 199 papers had negative treatments in 4 or more time bins; 2 papers published consistent harm in all timebins (*10, 11*). Patel et al., a systemically treated group, specifically the diet subcategory, reported negative treatment effects in all 7 time bins, and 89 out 136 data points (65.4%) from this paper had <1 normalized value (*10*) (Figure 8).

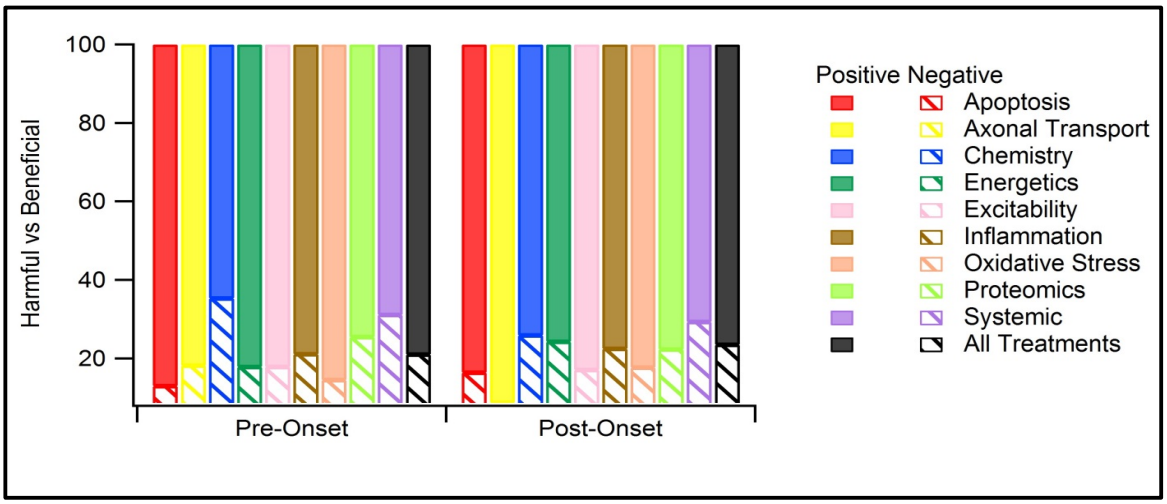


Figure 8 – Harmful treatments distribution by ontologies. While some treatment categories reported negative results more frequently than others, there was no meaningful pattern within the data.

1.19 Post-onset measure better than pre-onset measures

The normalized treatment effects of every category increased steadily throughout disease progression, with 121-130 or 131+ days being the most effective age groups. While this outcome is encouraging, considering that diagnosis and treatment would most likely not begin in an ALS patient pre-onset, these results are not unexpected given that as the disease progresses and the mices' health deteriorates, a treatment has more room for a drastic improvement in the assessment modalities included in this analysis (Figure 9).

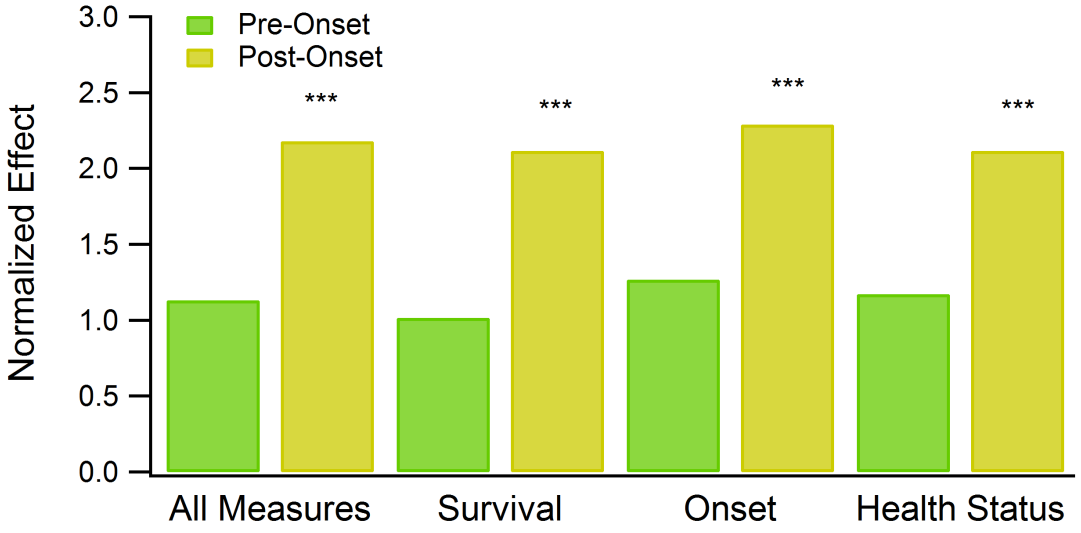


Figure 9 – Defining the onset threshold at 100 days, the average effect of all treatments compared to control yielded significantly more impact after onset than prior to in all assessment modalities. (*p < 0.0001).**

1.20 Combination treatments better than monotherapy

During pre-onset, the polytherapy treatments were not as effective as monotherapy (Figure 10). Post-onset, the combination treatments were becoming more effective than monotherapy treatments during the diseased states (111-120 and 121-130 days groups), and its efficacy at end-stage was statistically higher than monotherapy for all measures (p

< 0.05).

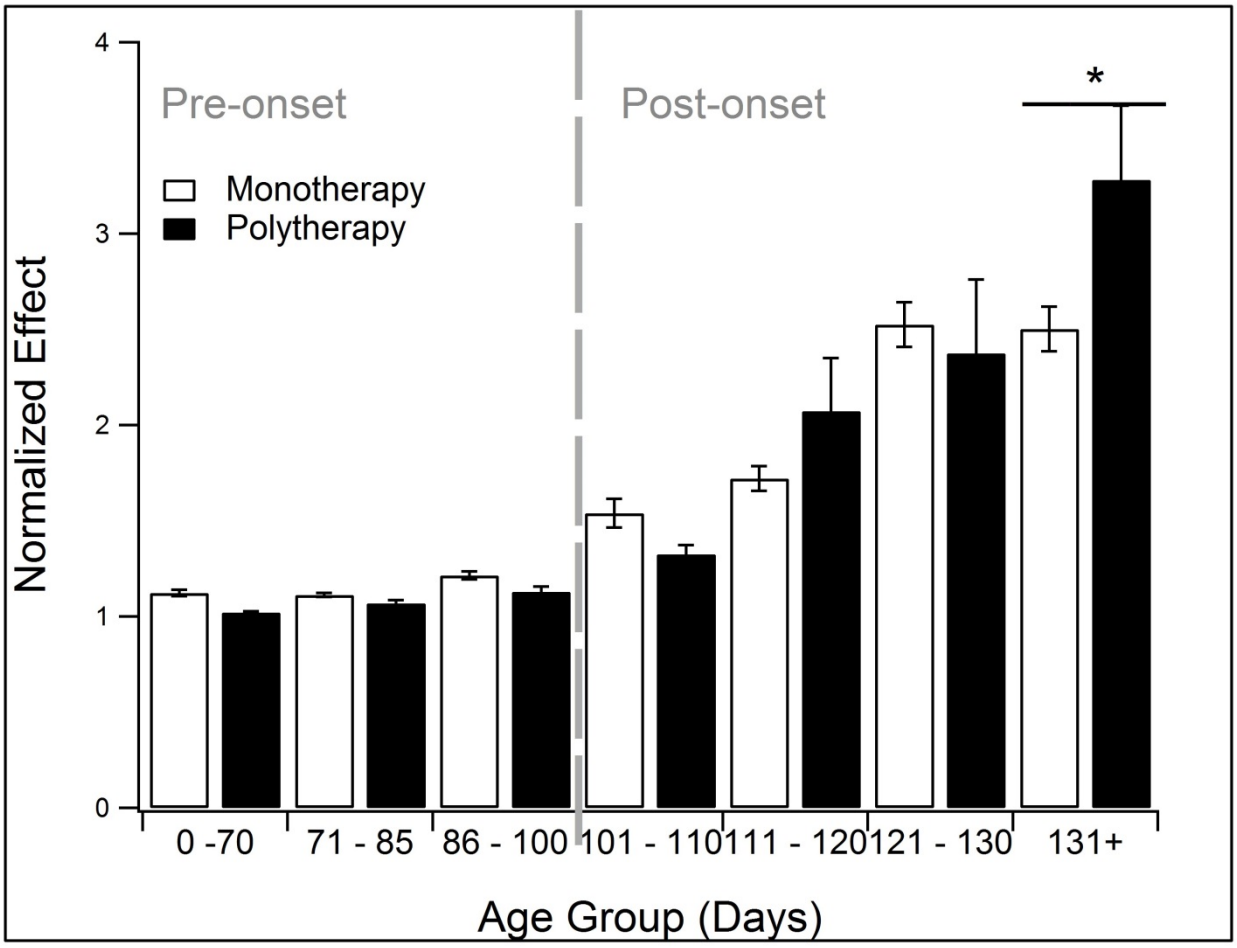


Figure 10 – Comparison of the treatment effects of monotherapy and polytherapy treatments. Polytherapy treatment effect started to climb around disease onset and performed significantly better than monotherapy at the end stage 131+ days age group. * $p < 0.05$.

DISCUSSION

1.21 "Best" treatment categories change with disease progression

Results from this meta-analysis of different treatment categories in high copy G93A SOD1 transgenic mouse models throughout the entirety of their lifespan revealed that specific treatment approaches performed better than others during different stages of disease progression.

1.21.1 Before Onset (0-100 days)

Overall no category of treatments performs better than the others in stages immediately pre-onset. It has been noted in literature that ALS patients have less antecedent disease than the average population, possibly due to the overcompensation for the aberrancy (*16*). Also, it has been noted that certain disease parameters, such as the oxidative stress level in transgenic mice do seem to improve briefly preceding the onset (*17*). Such findings align with the results that the untreated group may be performing almost as well as the control group for most of the treatments, since the mice must be physiologically overcompensating for the impending disease. Another interpretation of this result could be that all treatments produce similar positive feedback from mice that none of them is better than the other in comparison.

1.21.2 Onset (101-110 days)

Excitotoxicity treatments perform better at the onset. Glutamate-induced excitotoxicity resulting in motor neuron death is one of the pathogenic mechanisms in ALS. As mSOD1 increases the sensitivity of the AMPA receptor to glutamatergic stimulation

and disrupts mitochondrial function, affecting the surrounding astrocytes. Excitotoxicity treatments interrupt glutamatergic transmission and lower glutamate concentration, resulting in protection against motor neuron degeneration (*18*). There are excitability treatments known in literature to extend survival but not delay the onset, which is consistent with the findings of excitotoxicity cell death being a late phenomenon in mutant SOD1-mediated neurodegeneration (*19-23*). The results from this study, however, indicate the opposite: treatments in excitability category are effective for onset during this stage while the survival metrics were not noticeably different from those of the other treatments. However, most of anti-excitotoxicity treatment effects came from non-survival/onset measures, which is consistent with previous findings, among them the fact that ceftriaxone significantly delayed the decrease in the body weight and muscle strength (*19*).

1.21.3 After Onset (111 days of age and after)

1.21.3.1 Early Diseased Stage (111-120 days)

Treatments targeting axonal transport category are effective during the early diseased stage. Axonal transport is known to start being compromised very early in mice lifespan, around 20-50 days (*24, 25*). A study even found that axons can degenerate without preceding transport deficits and vice versa (*25*). While treatments in axonal transport category are known to prolong survival (*26, 27*), the mechanisms are unclear as to how they may extend the survival, especially during 111-120 days of age. Perhaps more data and studies in the treatment group, which originally had the smallest sample size of all categories, will give a clearer picture of the effects on disease progression.

1.21.3.2 Late Diseased Stage (121-130 days)

All treatment groups performed better during the late diseased stage. In this intermediate state between the symptomatic state and late stage, high copies G93A mice are having more severe symptoms and rapidly deteriorated health (*28*). Thus, the effect from treatment regimens would show a better performance than earlier stages when the data is normalized. However, at a closer look, inflammatory performed 16% better than the average treatment effect. Besides playing a major role in the pathogenesis of motor neuron death in ALS, neuroinflammation accelerates disease progression (*18*). Thus recently, interest in inflammatory treatments has been growing (*19*). It has been suggested that early anti-inflammatory treatment is necessary to interrupt ALS-induced neuroinflammation (*29*), and the promising outcomes of the immunoregulatory treatments from the early stage of the mice's lifespan are consistent with this postulation as inflammation category has a large number of pre-onset treatment.

1.21.3.3 Late Stage (131 days and after)

Anti-apoptotic and chemistry treatments performed significantly better during late stage (131 days and after). Any data from the time at which the untreated G93A mice would normally die were classified in the last bin in the analysis. Most treatments do show an effect at this point due to the increased longevity of the treated mice, but only the anti-apoptotic treatments perform significantly better than the other categories. This result is consistent with another review pointing out that agents that block apoptosis in ALS mice primarily affect disease endpoint (*19*). The anti-apoptotic treatments that were evaluated in this stage include zVAD-fmk, an enzymatic caspase inhibitor (*8*), AEOL 10150, a manganese porphyrin with anti-apoptotic properties (*9*), p75 knockout (*30*), melatonin (*2*), as well as other factors (Supplemental Table 1). However, some of this might be explained

by a greater number of studies classified as apoptotic being targeted at exploring the underlying mechanisms of ALS progression rather than testing prospective treatments, and may have limited predictive value. On the other hand, chemistry category had almost as great an effect as anti-apoptosis treatments, although not to a statistically significant extend due to its small sample size. Intracellular copper homeostasis was known not to be altered in the pre-symptomatic transgenic mice (*31*), and this may explain why treatments in chemistry category become effective only in the later stages of diseased mice life.

1.22 Within Systemic Category

Unconventional subcategory performed significantly well in early life (0 - 85 days). Interestingly, the category that includes unconventional treatments such as light therapy or castration outperformed the other well-defined and established and advanced approaches like gene therapy. A possible explanation could be that these treatments have more immediate effect than other subcategories like growth factors injection or gene therapy.

Growth factor subcategory performed better than others in immediate pre-onset stage (86 - 100 days) and symptomatic stage (111-130). With the direct effects on neuronal growth, axonal outgrowth and neuroprotection, growth factor subcategory treatments can specifically inhibit superoxide-induced cell death and promote neuronal survival by interacting with proteins having neurotrophic or neurotoxic properties (*32, 33*). With its prominent potential, growth factors have been the subject of multiple clinical trials for ALS treatment since the 1990s, but the results from these trials have been either slowed disease progression (*34*) or safe but no improvement or no difference (*35, 36*). Our results also found that this subcategory improves all measures throughout the symptomatic stage but

not during the last stage.

Cell transplantation has better effect during the onset stage (101 - 110 days). The cell transplantation approach has been gaining a lot of attention lately as a powerful tool for treating neurological disorders such as ALS. These therapies aimed at cell replacement or neuroprotection while stimulating patient's own repair mechanisms so that the transplanted stem cells become integrated into the system and infiltrate the areas affected by disease (*37*). In the ALS case, using stem cell could prevent motor neurons from dying as they migrate into the spinal cord and induce motor recovery (*38*). A clinical trial has found mesenchymal stem cells transplantation in ALS to be safe and well-tolerated in 26 patients and at 6 month after treatment, 80% of the patients improved by more than 35% and 67% by more than 50% (*39*).

During the late stage (141 days onward), diet and exercise became more effective. While diet and exercise are known to improve physiological parameters in completely normal mice (and human beings), and it may be the reason why the mice that had not developed symptoms still showed treatment effects. However, in some studies, the manipulations of exercise and dietary restrictions resulted in conflicting and fluctuating results. Dietary restriction was reported to improve motor performance but significantly worsen the ultimate outcome in mice by hastening disease onset (*11*). Low and moderate treadmill exercise was beneficial in transgenic mice although with opposing gender effects (*40, 41*), while high intensity exercise was either harmful or had no significant effect (*42, 43*).

1.23 Combination therapy is the future of ALS treatment

Comprising 7% (567 / 7607) of the data in this analysis, combination therapy were treatments categorized into more than one ontology. This subgroup is significantly more beneficial to the mice during the later disease stage of 123-140 days (Figure 6). This is consistent with previous literature demonstrating that combination treatments have improved disease metrics more effectively than either of the treatments conducted alone. A synergistic approach to such a complex disease would be more likely to address all the varied factors involved in the ALS instability.

1.24 Treatments administered pre-onset show most benefit post-onset

Most treatments in this meta-analysis were most effective at later disease stages than earlier. This late benefit could be due to the fact that in transgenic mice, ALS symptoms and body condition degradation are exacerbated in later stages, so there is naturally a higher chance for dramatic improvements. Interestingly, most of the studies also began administering treatments prior to onset, so the benefit to the mice was a delayed outcome. This result correlates with many recent studies indicating pretreating at-risk patients had the best results for various neurological diseases. Because of these promising results, future experiments are being designed to test the effect of pretreating patients prior to disease onset. One ongoing study aims to test the efficacy of drug administration before disease onset on a family in Antioquia, Colombia whose rare gene mutation guarantees their development of early Alzheimer's disease (51). Only a few Alzheimer's patients benefitted from the drug so far, but it is believed the initial time of treatment was potentially

too late. Nevertheless, pretreating patients prior to onset has been studied in various diseases (52-54) and future research will expand upon this proactive approach idea.

1.25 Modality assessment can distort apparent treatment efficacy

As demonstrated by the assessment modality analyses, certain ontologies are particularly effective in delaying onset, prolonging survival, or improving quality of life health status measures, but not all three. For example, inflammation and apoptosis were most effective at delaying onset at differing disease stages. During early life, neuroinflammation may be the most aggravating instability that accelerates ALS progression (*18*), but seems to be less important later on as other issues arise. While research has shown that blocking apoptosis towards end-stage should be a primary target (*19*), this seems to do nothing to prolong survival, but will reduce onset characteristics and improve the health measures in the mice (Figure 4). Conversely, treatments targeting axonal transport were most effective in delaying survival only; there could potentially be more benefit to this targeted approach, but it is the least researched in the field, and the lack of data excluded this ontology from some of the analysis. Axonal transport slowing occurs very early in the disease progression (*55*), with the anterograde mitochondrial transport most affected by the SOD1 mutation. Detecting and immediately treating these issues during early-onset should be a priority (*56*), and could continue to be beneficial later in life (Figure 4B).

Treatments may be more beneficial in improving only survival or onset, but given the disparity in treatment efficacy for these specific assessments compared to the health measures modality, how the clinical progression of ALS is tracked will affect results. The

health measures analysis included the standard tracking parameters: rotarod performance, grip strength, body weight, etc., as well as any other assessment used, but the effectiveness of these in monitoring the mice has been debated (*57*). Further research into which assessments are more reliable in determining onset and tracking disease progression may help standardize treatment evaluations.

1.26 Not all intuitive treatments are helpful.

Epidemiological studies indicate that multiple environmental agents are responsible for triggering ALS (*58*). Due to the complexity of ALS, some treatments were intended to improve, yet they resulted negatively in disease measures (Figure 8). For example, one included study tested the effect of caloric restriction, which hypothetically increases mitochondrial oxidative capacity. However, increasing mitochondrial oxidative capacity could be only observed in female G93A mice not male G93A mice (*10*). 65.4% of results after caloric restriction treatment had <1 normalized values, and they could have been found in all 7 time bins, which indicates that caloric restriction treatment was a harmful treatment. According to the result of this study, not all intuitive treatments benefit ALS, so treatments should be chosen carefully and examined in the context of the entire physiology and disease timeline.

CONCLUSION

This meta-analysis was conducted in order to aggregate existing published data on the comparative benefits of various treatment options in the SOD1 G93A transgenic mouse. By determining the most promising pathophysiological approaches, future research can be more focused on the best treatments and advance the field at a further rate. The results have determined that there is a temporal relationship between treatment approach and disease progression, which supports the theory of combinatorial medicine to match the complexity of a neurodegenerative disease. It also demonstrates that starting a treatment before disease onset will yield benefits throughout the rest of disease progression all the way to end-stage, which could have an impact on those patients that develop familial ALS. Hopefully future research will yield an accurate predictive model to anticipate the dominant instability within an ALS patient and select the appropriate response to develop a personalized treatment regimen.

REFERENCES

1. R. G. Miller, J. D. Mitchell, D. H. Moore, Riluzole for amyotrophic lateral sclerosis (ALS)/motor neuron disease (MND). *Cochrane Database Syst Rev*, CD001447 (2012).
2. Y. Zhang *et al.*, Melatonin inhibits the caspase-1/cytochrome c/caspase-3 cell death pathway, inhibits MT1 receptor loss and delays disease progression in a mouse model of amyotrophic lateral sclerosis. *Neurobiol Dis* **55**, 26-35 (2013).
3. A. Gianforcaro, J. A. Solomon, M. J. Hamadeh, Vitamin D(3) at 50x AI attenuates the decline in paw grip endurance, but not disease outcomes, in the G93A mouse model of ALS, and is toxic in females. *PLoS One* **8**, e30243 (2013).
4. E. J. Yang *et al.*, Bee venom attenuates neuroinflammatory events and extends survival in amyotrophic lateral sclerosis models. *J Neuroinflammation* **7**, 69 (2010).
5. R. B. Kim, C. W. Irvin, K. R. Tilva, C. S. Mitchell, State of the field: An informatics-based systematic review of the SOD1-G93A amyotrophic lateral sclerosis transgenic mouse model. *Amyotroph Lateral Scler Frontotemporal Degener* **17**, 1-14 (2015).
6. S. R. Pfohl, M. T. Halicek, C. S. Mitchell, Characterization of the Contribution of Genetic Background and Gender to Disease Progression in the SOD1 G93A Mouse Model of Amyotrophic Lateral Sclerosis: A Meta-Analysis. *J Neuromuscul Dis* **2**, 137-150 (2015).
7. C. S. Mitchell, A. Cates, R. B. Kim, S. K. Hollinger, Undergraduate Biocuration: Developing Tomorrow's Researchers While Mining Today's Data. *J Undergrad Neurosci Educ* **14**, A56-65 (2015).
8. M. Li *et al.*, Functional role of caspase-1 and caspase-3 in an ALS transgenic mouse model. *Science* **288**, 335-339 (2000).
9. S. Petri *et al.*, Additive neuroprotective effects of a histone deacetylase inhibitor and a catalytic antioxidant in a transgenic mouse model of amyotrophic lateral sclerosis. *Neurobiol Dis* **22**, 40-49 (2006).
10. B. P. Patel, A. Safdar, S. Raha, M. A. Tarnopolsky, M. J. Hamadeh, Caloric restriction shortens lifespan through an increase in lipid peroxidation, inflammation and apoptosis in the G93A mouse, an animal model of ALS. *PLoS One* **5**, e9386 (2010).
11. M. J. Hamadeh, M. C. Rodriguez, J. J. Kaczor, M. A. Tarnopolsky, Caloric restriction transiently improves motor performance but hastens clinical onset of disease in the Cu/Zn-superoxide dismutase mutant G93A mouse. *Muscle Nerve* **31**, 214-220 (2005).
12. A. E. Renton, A. Chio, B. J. Traynor, State of play in amyotrophic lateral sclerosis genetics. *Nat Neurosci* **17**, 17-23 (2014).
13. X. Paez-Colasante, C. Figueroa-Romero, S. A. Sakowski, S. A. Goutman, E. L. Feldman, Amyotrophic lateral sclerosis: mechanisms and therapeutics in the epigenomic era. *Nat Rev Neurol* **11**, 266-279 (2015).

14. E. F. Goodall, K. E. Morrison, Amyotrophic lateral sclerosis (motor neuron disease): proposed mechanisms and pathways to treatment. *Expert Rev Mol Med* **8**, 1-22 (2006).
15. S. K. Hollinger, I. S. Okosun, C. S. Mitchell, Antecedent Disease and Amyotrophic Lateral Sclerosis: What Is Protecting Whom? *Front Neurol* **7**, 47 (2016).
16. C. S. Mitchell *et al.*, Antecedent Disease is Less Prevalent in Amyotrophic Lateral Sclerosis. *Neurodegener Dis* **15**, 109-113 (2015).
17. C. W. Irvin, R. B. Kim, C. S. Mitchell, Seeking homeostasis: temporal trends in respiration, oxidation, and calcium in SOD1 G93A Amyotrophic Lateral Sclerosis mice. *Front Cell Neurosci* **9**, 248 (2015).
18. R. S. Pandya *et al.*, Therapeutic neuroprotective agents for amyotrophic lateral sclerosis. *Cell Mol Life Sci* **70**, 4729-4745 (2013).
19. B. J. Turner, K. Talbot, Transgenics, toxicity and therapeutics in rodent models of mutant SOD1-mediated familial ALS. *Prog Neurobiol* **85**, 94-134 (2008).
20. R. Ganel *et al.*, Selective up-regulation of the glial Na+-dependent glutamate transporter GLT1 by a neuroimmunophilin ligand results in neuroprotection. *Neurobiol Dis* **21**, 556-567 (2006).
21. G. D. Ghadge *et al.*, Glutamate carboxypeptidase II inhibition protects motor neurons from death in familial amyotrophic lateral sclerosis models. *Proc Natl Acad Sci U S A* **100**, 9554-9559 (2003).
22. J. D. Rothstein *et al.*, Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. *Nature* **433**, 73-77 (2005).
23. P. Van Damme, M. Leyssen, G. Callewaert, W. Robberecht, L. Van Den Bosch, The AMPA receptor antagonist NBQX prolongs survival in a transgenic mouse model of amyotrophic lateral sclerosis. *Neurosci Lett* **343**, 81-84 (2003).
24. L. G. Bilsland *et al.*, Deficits in axonal transport precede ALS symptoms in vivo. *Proc Natl Acad Sci U S A* **107**, 20523-20528 (2010).
25. P. Marinkovic *et al.*, Axonal transport deficits and degeneration can evolve independently in mouse models of amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A* **109**, 4296-4301 (2012).
26. P. Fanara *et al.*, Stabilization of hyperdynamic microtubules is neuroprotective in amyotrophic lateral sclerosis. *J Biol Chem* **282**, 23465-23472 (2007).
27. K. Venkova *et al.*, Semaphorin 3A signaling through neuropilin-1 is an early trigger for distal axonopathy in the SOD1G93A mouse model of amyotrophic lateral sclerosis. *J Neuropathol Exp Neurol* **73**, 702-713 (2014).
28. M. E. Gurney *et al.*, Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science* **264**, 1772-1775 (1994).
29. A. Jeyachandran, B. Mertens, E. A. McKissick, C. S. Mitchell, Type I Vs. Type II Cytokine Levels as a Function of SOD1 G93A Mouse Amyotrophic Lateral Sclerosis Disease Progression. *Front Cell Neurosci* **9**, 462 (2015).
30. B. M. Kust, N. Brouwer, I. J. Mantingh, H. W. Boddeke, J. C. Copray, Reduced p75NTR expression delays disease onset only in female mice of a transgenic model of familial amyotrophic lateral sclerosis. *Amyotroph Lateral Scler Other Motor Neuron Disord* **4**, 100-105 (2003).

31. E. Tokuda, E. Okawa, S. Ono, Dysregulation of intracellular copper trafficking pathway in a mouse model of mutant copper/zinc superoxide dismutase-linked familial amyotrophic lateral sclerosis. *J Neurochem* **111**, 181-191 (2009).
32. A. Sanchez, S. Wadhwani, P. Grammas, Multiple neurotrophic effects of VEGF on cultured neurons. *Neuropeptides* **44**, 323-331 (2010).
33. C. B. Thompson, Apoptosis in the pathogenesis and treatment of disease. *Science* **267**, 1456 (1995).
34. E. C. Lai *et al.*, Effect of recombinant human insulin-like growth factor-I on progression of ALS. A placebo-controlled study. The North America ALS/IGF-I Study Group. *Neurology* **49**, 1621-1630 (1997).
35. G. D. Borasio *et al.*, A placebo-controlled trial of insulin-like growth factor-I in amyotrophic lateral sclerosis. European ALS/IGF-I Study Group. *Neurology* **51**, 583-586 (1998).
36. E. J. Sorenson *et al.*, Subcutaneous IGF-1 is not beneficial in 2-year ALS trial. *Neurology* **71**, 1770-1775 (2008).
37. J. A. Steinbeck, L. Studer., Moving stem cells to the clinic: potential and limitations for brain repair. *Neuron* **86**, 187-206 (2015).
38. D. A. Kerr *et al.*, Human embryonic germ cell derivatives facilitate motor recovery of rats with diffuse motor neuron injury. *J Neurosci* **23**, 5131-5140 (2003).
39. P. Petrou *et al.*, Safety and Clinical Effects of Mesenchymal Stem Cells Secreting Neurotrophic Factor Transplantation in Patients With Amyotrophic Lateral Sclerosis: Results of Phase 1/2 and 2a Clinical Trials. *JAMA Neurol* **73**, 337-344 (2016).
40. I. G. Kirkinezos, D. Hernandez, W. G. Bradley, C. T. Moraes, Regular exercise is beneficial to a mouse model of amyotrophic lateral sclerosis. *Ann Neurol* **53**, 804-807 (2003).
41. J. H. Veldink *et al.*, Sexual differences in onset of disease and response to exercise in a transgenic model of ALS. *Neuromuscul Disord* **13**, 737-743 (2003).
42. D. Liebetanz, K. Hagemann, F. von Lewinski, E. Kahler, W. Paulus, Extensive exercise is not harmful in amyotrophic lateral sclerosis. *Eur J Neurosci* **20**, 3115-3120 (2004).
43. D. J. Mahoney, C. Rodriguez, M. Devries, N. Yasuda, M. A. Tarnopolsky, Effects of high-intensity endurance exercise training in the G93A mouse model of amyotrophic lateral sclerosis. *Muscle Nerve* **29**, 656-662 (2004).
44. S. Waibel, A. Reuter, S. Malessa, E. Blaugrund, A. C. Ludolph, Rasagiline alone and in combination with riluzole prolongs survival in an ALS mouse model. *J Neurol* **251**, 1080-1084 (2004).
45. P. Klivenyi, M. Kiaei, G. Gardian, N. Y. Calingasan, M. F. Beal, Additive neuroprotective effects of creatine and cyclooxygenase 2 inhibitors in a transgenic mouse model of amyotrophic lateral sclerosis. *J Neurochem* **88**, 576-582 (2004).
46. J. H. Shin *et al.*, Concurrent administration of Neu2000 and lithium produces marked improvement of motor neuron survival, motor function, and mortality in a mouse model of amyotrophic lateral sclerosis. *Mol Pharmacol* **71**, 965-975 (2007).

47. J. Kriz, G. Gowing, J. P. Julien, Efficient three-drug cocktail for disease induced by mutant superoxide dismutase. *Ann Neurol* **53**, 429-436 (2003).
48. P. H. Gordon *et al.*, A novel, efficient, randomized selection trial comparing combinations of drug therapy for ALS. *Amyotroph Lateral Scler* **9**, 212-222 (2008).
49. E. Louvel, J. Hugon, A. Doble, Therapeutic advances in amyotrophic lateral sclerosis. *Trends Pharmacol Sci* **18**, 196-203 (1997).
50. S. P. Aggarwal *et al.*, Safety and efficacy of lithium in combination with riluzole for treatment of amyotrophic lateral sclerosis: a randomised, double-blind, placebo-controlled trial. *Lancet Neurol* **9**, 481-488 (2010).
51. F. Lopera *et al.*, Clinical features of early-onset Alzheimer disease in a large kindred with an E280A presenilin-1 mutation. *Jama* **277**, 793-799 (1997).
52. K. Kajinami *et al.*, Effect of pretreatment vitamin D levels on in vivo effects of atorvastatin on bone metabolism in patients with heterozygous familial hypercholesterolemia. *Am J Cardiol* **92**, 1113-1116 (2003).
53. M. Jansen *et al.*, Direct adsorption of lipoproteins (DALI) from whole blood: first long-term clinical experience with a new LDL-apheresis system for the treatment of familial hypercholesterolaemia. *Wien Klin Wochenschr* **112**, 61-69 (2000).
54. G. Artom *et al.*, Pretreatment with corticosteroids attenuates the efficacy of colchicine in preventing recurrent pericarditis: a multi-centre all-case analysis. *Eur Heart J* **26**, 723-727 (2005).
55. T. L. Williamson, D. W. Cleveland, Slowing of axonal transport is a very early event in the toxicity of ALS-linked SOD1 mutants to motor neurons. *Nat Neurosci* **2**, 50-56 (1999).
56. K. J. De Vos *et al.*, Familial amyotrophic lateral sclerosis-linked SOD1 mutants perturb fast axonal transport to reduce axonal mitochondria content. *Hum Mol Genet* **16**, 2720-2728 (2007).
57. P. Weydt, S. Y. Hong, M. Kliot, T. Moller, Assessing disease onset and progression in the SOD1 mouse model of ALS. *Neuroreport* **14**, 1051-1054 (2003).
58. A. Eisen, Amyotrophic lateral sclerosis is a multifactorial disease. *Muscle Nerve* **18**, 741-752 (1995).
59. R. Mancuso *et al.*, Lack of synergistic effect of resveratrol and sigma-1 receptor agonist (PRE-084) in SOD1G(9)(3)A ALS mice: overlapping effects or limited therapeutic opportunity? *Orphanet J Rare Dis* **9**, 78 (2014).